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ORIGINAL ARTICLE

Overexpression of Cannabinoid CB2 Receptor in the Brain Induces Hyperglycaemia and a Lean Phenotype in Adult Mice

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It is well known that the endocannabinoid system, through cannabinoid CB1 receptor activation, has an important role in the main aspects of energy balance (i.e. food intake, energy expenditure and glucose and fat metabolism), orchestrating all the machinery involved in body weight control and energy homeostasis. A number of studies have revealed a crucial role of brain CB1 receptors in these processes. However, functional cannabinoid CB2 receptors have also been described in the brain, with no studies addressing their putative role in body weight control and glucose homeostasis. We have tested this hypothesis by analysing fasting-induced feeding, body weight, some hypothalamic neuropeptides, glucose tolerance and plasma hormones in an animal model specifically overexpressing CB2 receptors in the central nervous system. We found that specific overexpression of CB2 receptors in the brain promoted higher basal glucose levels, decreased fasting-induced feeding and, eventually, led to a lean phenotype and glucose intolerance. These findings could not be attributed to decreased locomotor activity, increased anxiety or depressive-like behaviours. The expression of relevant neuropeptides such as pro-opiomelanocortin and galanin in the arcuate nucleus of the hypothalamus was altered but not those of the CB1 receptor. Indeed, no changes in CB1 expression were found in the liver, skeletal muscle and adipose tissue. However, cannabinoid CB1 and CB2 receptor expression in the endocrine pancreas and glucagon plasma levels were decreased. No changes in plasma adiponectin, leptin, insulin and somatostatin were found. Taken together, these results suggest a role for central cannabinoid CB2 receptors in body weight control and glucose homeostasis.

Key words: *Cnr2*, CNS, glucose homeostasis, diabetes, body weight.

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It is well known that the endocannabinoid system (ECS) has an important role in the main aspects of energy homeostasis (i.e. food intake, energy expenditure and glucose and fat metabolism), orchestrating all the machinery in order to balance body weight and maintain an adequate storage of fuel (1). Furthermore, it is well established that signalling through the ECS is altered in pathological conditions such as obesity, type 2 diabetes and the metabolic syndrome (2–4). All these previous studies have demonstrated the cannabinoid CB1 receptor to play a role in both normal and pathological conditions associated with energy balance. However, the putative contribution of cannabinoid CB2 receptor to energy balance and metabolism is less understood.

Cannabinoid CB2 receptors were first described in immune system cells, being involved in modulating immune responses (5). However, subsequent analysis revealed that this receptor is also expressed in organs controlling metabolism such as the liver (6), adipose tissue (7), skeletal muscle (8) and the endocrine pancreas (9, 10). In keeping, changes in their expression have been reported in obesity in both the liver and the adipose tissue (11), and CB2 specific activation potentiated inflammatory markers, insulin resistance and liver steatosis in

obese mice, whereas its blockade did the opposite (11). Indeed, animals genetically lacking the CB2 receptor in the whole body have been reported to increase food intake and body weight with age, unexpectedly displaying less insulin resistance and inflammatory markers than the wild-type (WT) (12). These mice lacking the CB2 receptor were also more resistant to the development of diet-induced obesity (12).

Interestingly, the cannabinoid CB2 receptor has been also detected in several brain areas, such as the brainstem, cerebellum, cortex and hippocampus (13, 14). At least at the brainstem level, these receptors have been shown to be functional, being involved in the inhibition of emesis (13). However, the putative role of central cannabinoid CB2 receptors in modulating body weight and glucose homeostasis has not yet been explored. In a first approach to this issue, we developed a genetically-modified animal model that overexpress the CB2 receptor in the central nervous system (CNS) and analysed several parameters related to food intake and glucose handling. Briefly, we found that overexpression of CB2 receptor in the brain, including areas typically involved in energy homeostasis, promoted hyperglycaemia, decreased fasting-induced feeding and eventually led to the development of a lean phenotype that could not be attributed to behavioural alterations, with concomitant hyperglycaemia and decreased glucose tolerance. Indeed, central cannabinoid CB2 receptor overexpression decreased the expression of relevant neuropeptides such as pro-opiomelanocortin (POMC) and galanin in the arcuate nucleus (ARC) of the hypothalamus and had no impact on metabolic plasma hormones such as adiponectin, leptin, insulin or somatostatin. However, we detected a decrease in plasma glucagon levels and CB2 receptor expression in the endocrine pancreas. Taken together, these results suggest a role for central cannabinoid CB2 receptors in energy homeostasis.

Materials and methods

CB2xP mice generation and characterisation

Adult male mice overexpressing cannabinoid CB2 receptors (CB2xP) and WT littermates were used in all experiments. Transgenic mice overexpressing the murine CB2 receptor were developed in Dr J. Manzanares's laboratory, as described previously (15). These transgenic mice were generated by using a murine PrP promoter that is specifically expressed in neurones and glial cells (16). Male Swiss albino ICR mice (WT) were purchased from Harlan (Harlan, Barcelona, Spain). The CB2xP mice and WT mice used in a given experiment originated from the same breeding series and were matched for age and weight. Mice were maintained under a 12 : 12 h light/dark cycle (lights on 08.00 h) at a constant temperature of 21 ± 2 °C and 55 ± 5% relative humidity, with free access to food and water. All experimental procedures were carried out according with the highest standards of animal care, monitoring health, and minimizing pain and suffering, in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and Spanish national law on the care and use of laboratory animals. CB2 gene expression in discrete brain areas and behavioural characterisation of CB2xP versus WT was performed as described previously (17, 18).

Immunohistochemistry

The presence and relative abundance of CB1 and CB2 receptors in the hypothalamus, skeletal muscle (abdominal), liver, visceral white adipose tissue and pancreas from WT and CB2xP mice (n = 5 mice each group) was analysed by immunohistochemistry and densitometry. These tissues were fixed in 4% paraformadehyde in phosphate-buffered saline (PBS) by immersion, dehydrated and embedded in paraffin. Tissue blocks were cut into 5 μm-thick sections using a Microm HM325 microtome (Microm, Walldorf, Germany). To obtain matched immunohistochemistry from both genotypes, two replicates from two samples (each sample belonging to different group) were mounted on the same glass slide. Sections were dewaxed, washed several times with PBS, and incubated in 3% hydrogen peroxide in PBS for 20 min in the dark at room temperature to inactivate endogenous peroxidase. After three washes in PBS for 5 min, antigen retrieval was achieved by incubating in sodium citrate (pH 9) for 5 min at 80 °C. A background blocker solution containing 10% donkey serum, 0.3% Triton X-100 and 0.1% sodium azide was used to incubate the sections for 1 h, which was followed by incubation overnight at room temperature with the primary antibody : rabbit polyclonal CB1 and CB2 antibody (diluted 1 : 100 and 1 : 50, respectively; ABR catalogue no. PA1-745 and PA1-746A; Affinity Bioreagents, Golden, CO, USA). Antibody specificity was assessed by immunostaining paraffin-embedded pancreatic sections from CB1 and CB2 knockout (KO) mice. After antibody incubation, sections were washed three times with PBS, incubated in a biotinylated donkey anti-rabbit immunoglobulin G (Amersham, Little Chalfont, UK) diluted 1 : 500 for 1 h, washed again in PBS, and incubated in ExtrAvidin peroxidase (Sigma, St Louis, MO, USA) diluted 1 : 2000 in darkness at room temperature for 1 h. After three washes in PBS in darkness, we revealed immunolabeling with 0.05% diaminobenzidine (Sigma), 0.05% nickel ammonium sulphate and 0.03% H_2O_2 in PBS. All steps were carried out by gentle agitation at room temperature. Sections were dehydrated in ethanol, cleared in xylene and coverslipped with Eukitt mounting medium (Kindler GmBH and Co., Freiburg, Germany). Digital high-resolution microphotographs of the samples were taken under the same conditions of light and brightness/contrast by an Olympus BX41 microscope equipped with a 10× objective and an Olympus DP70 digital camera (Olympus Europa GmbH, Hamburg, Germany). Quantification of immunoreactivity was carried out by measuring densitometry of the images obtained from each replicate and sample: two replicates per sample, five samples per group and two groups, using the analysis software IMAGEJ (National Institute of Health, Bethesda, MD, USA).

Glucose tolerance test (GTT) and plasma determinations

Glucose tolerance was investigated in 12-h fasted, 12-week-old, WT, CB2xP and CB2 KO mice acutely treated with cannabinoid drugs [the endocannabinoid anandamide (AEA), the CB2 specific agonist JWH133 and the CB2 specific antagonist AM630] and in untreated 12-, 15-, 18- and 24-week-old mice from WT and CB2xP genotypes. Cannabinoid drugs were i.p. injected 30 min before the GTT. All the drugs were purchased from Tocris (Tocris Bioscience, Bristol, UK) and diluted in 0.1% dimethylsulphate–5% Tween 80 in saline. The doses were selected based on previous studies showing changes in glucose tolerance after these treatments in rats (19, 20). Animals were moved to the experimental room 30 min before procedure and the GTT was carried out by injecting an i.p. glucose load of 2 g/kg body weight (diluted in saline). Tail blood samples were collected before (0 min) and 5, 10, 15, 30, 60 and 120 min after glucose administration. Glucose was determined using a commercial glucometer (Accu-check; Roche Diagnostic, Barcelona, Spain). For measuring plasma hormones, 15- and 24-week-old WT and CB2xP mice were fasted for 12 h and tail blood samples were collected in ethylenediaminetetraacetic acid-treated tubes, centrifuged at 2000 *g* for 10 min at 4 °C, and analysed by enzyme-linked immunosorbent assay kits for adiponectin (B-Bridge International, Inc., Cupertino, CA, USA), leptin (B-Bridge International, Inc.), insulin (Mercodia, Uppsala, Sweden), glucagon (Gentaur Europe BVBA, Brussels, Belgium) and somatostatin (Bachem AG, Bubendorf, Switzerland) in accordance with the manufacturer's instructions.

Table 1. Primers Sequences used for Quantifying mRNA Expression in the Arcuate Nucleus of the Hypothalamus.

Gene symbol (name)	Oligosense (5′ → 3′) Oligoantisense (5′ → 3′)	GenBank® accession number	Product size (bp)	Annealing temperature (°C)
Gal	GAGACCAGGAAGTGTTGATGTG GACGATTGGCTTGAGGAGTT	NM_010253.3	250	56.6
Npy	GCTCTGCGACACTACATCAA TGGTTTCAGGGGATGAGATG	NM_023456.2	214	54.8
Pomc	CTCCTGCTTCAGACCTCCA TTTTCAGTCAGGGGCTGTTC	NM_008895.3	173	59.8
Cnr2	TGCCCAAAGGAACTAACAGG CCTCCCAAACAGCGATTAGA	NM_009924.3	215	54.4
Pck1	QT00153013[a]	NM_011044.2	93	60.0
G6pc	QT00114625[a]	NM_008061.3	99	60.0
H2ab	CCGTGCTGGAGTACCTGACG ATTACTTCCCCTTGGCCTTG	NM_175660.2	234	64.0

[a] QuantiTect Primer Assay (Qiagen Inc.).

Glucose and insulin area under the curve (AUC) was calculated from their corresponding graphs by using IMAGEJ software.

Food intake measurement in 12-h food-deprived mice

Male mice belonging to both genotypes, 10–12 weeks old (n = 8 each genotype), were individually housed 2 days before the experiment and fasted for 12 h just before starting the experimental procedure. Food weight was monitored at 30, 60, 120 and 240 min after food presentation and relative to weight cumulative food intake was calculated.

Quantitative real-time polymerase chain reaction (RT-PCR)

Twelve-week- and 24-week-old WT and CB2xP mice (n = 8 each genotype) were killed by cervical dislocation. The brains and liver were extracted and micropunches were obtained from the different brain areas to be analysed, snap frozen and stored at −80 °C for further analysis. Total RNA was isolated from these areas, using Biozol reagent (bioWORLD, Dublin, OH, USA) in accordance with the manufacturer's instructions. RNA was then purified using the Qiagen RNeasy Mini Kit and treated with DNase I (4 U for each RT reaction) for 30 min at 25 °C before reverse transcription (Qiagen Inc., Valencia, CA, USA). RNA quality was assessed on a 1% agarose gel and by optical density measurement, with all the samples displaying the 28S and 18S fragments and an A_{260}/A_{280} ratio > 1.8. Total purified RNA (1 μg) was reversed transcribed into complementary DNA (cDNA) using a transcriptor reverse transcriptase kit (Roche Applied Science, Indianapolis, IN, USA) in accordance with the manufacturer's instructions. Negative control included RT reactions omitting reverse transcriptase. The obtained cDNA was used as the template for the quantitative RT-PCR that was performed in an iCycler system (Bio-Rad, Hercules, CA, USA) using the QuantiTect SYBR Green Master (Rox) kit (Roche Diagnostics, Indianapolis, IN, USA). Cycling parameters were 95 °C for 10 min to activate the HotStart DNA polymerase, then 40 cycles of 95 °C for 15 s, annealing temperature (Table 1) for 40 s, and a

Table 2. Mean Relative Expression of CB2 mRNA in CB2xP.

Brain regions	mRNA over-expression levels (%)	Statistical test (Student's t-test)
Acc	180	t = −4.590, P = 0.001, d.f. = 9
Cg	199	t = −6.125, P < 0.001, d.f. = 9
Amy	64	t = −1.573, P = 0.05, d.f. = 9
Hipp	158	t = −2.464, P = 0.027, d.f. = 14
VMN	126	t = −2.863, P < 0.001, d.f. = 9
ARC	157	t = −2.184, P = 0.05, d.f. = 9
VTA	100	t = 3.876, P = 0.003, d.f. = 10

Twelve-week-old CB2xP mice exhibited significantly increased CB2 receptor mRNA levels in all the regions analysed compared to wild-type mice. Relative expression was calculated using the C_t method. Acc, nucleus accumbens; Amy, amygdala; ARC, arcuate nucleus of the hypothalamus; Cg, cingulated cortex; Hipp, hippocampus; VMN, ventromedial nucleus of hypothalamus; VTA, ventral tegmental area.

Table 3. Behavioural Characterisation of CB2xP Versus Wild-Type (WT): Summary of Previously Published Behavioural Characterisation Regarding Anxiety and Depression in the CB2 Receptor Overexpressing Mice (17, 22).

Test	Results
Open field	No differences were found between CB2xP and WT mice in the total distance travelled. However, the central distance travelled increased significantly and the peripheral distance travelled decreased significantly in CB2xP mice
Light dark box	CB2xP mice spent significantly more time in the light box
Elevated plus maze	CB2xP mice displayed a significantly increased the time spent in the open arms
Tail suspension	CB2xP mice showed significantly less duration of immobility compared to WT
Novelty suppressed feeding (NSFT)	CB2xP mice presented a significantly shorter latency and increased consumption of food pellets (g) compared to WT mice during NSFT
Chronic mild stress (CMS)	CMS failed to produce any alteration in the CB2xP mice in the TST and sucrose consumption. In addition, no changes were observed in BDNF gene and protein expression in stressed CB2xP mice
Evaluation of CRF and POMC gene expressions under basal and restraint stress conditions	In WT mice, restraint stress markedly increased CRF (82%) and POMC (42%) gene expression in the PVN and ARC nucleus of the hypothalamus, respectively. By contrast, restraint stress failed to produce any alteration in CB2xP mice and reduced the increase (22%) in POMC gene expression

Twelve-week-old CB2xP mice displayed no depressive symptoms or anxiogenic-like behaviours. ARC, arcuate nucleus; BDNF, brain-derived neurotrophic factor; CRF, corticotrophin-releasing factor; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus.

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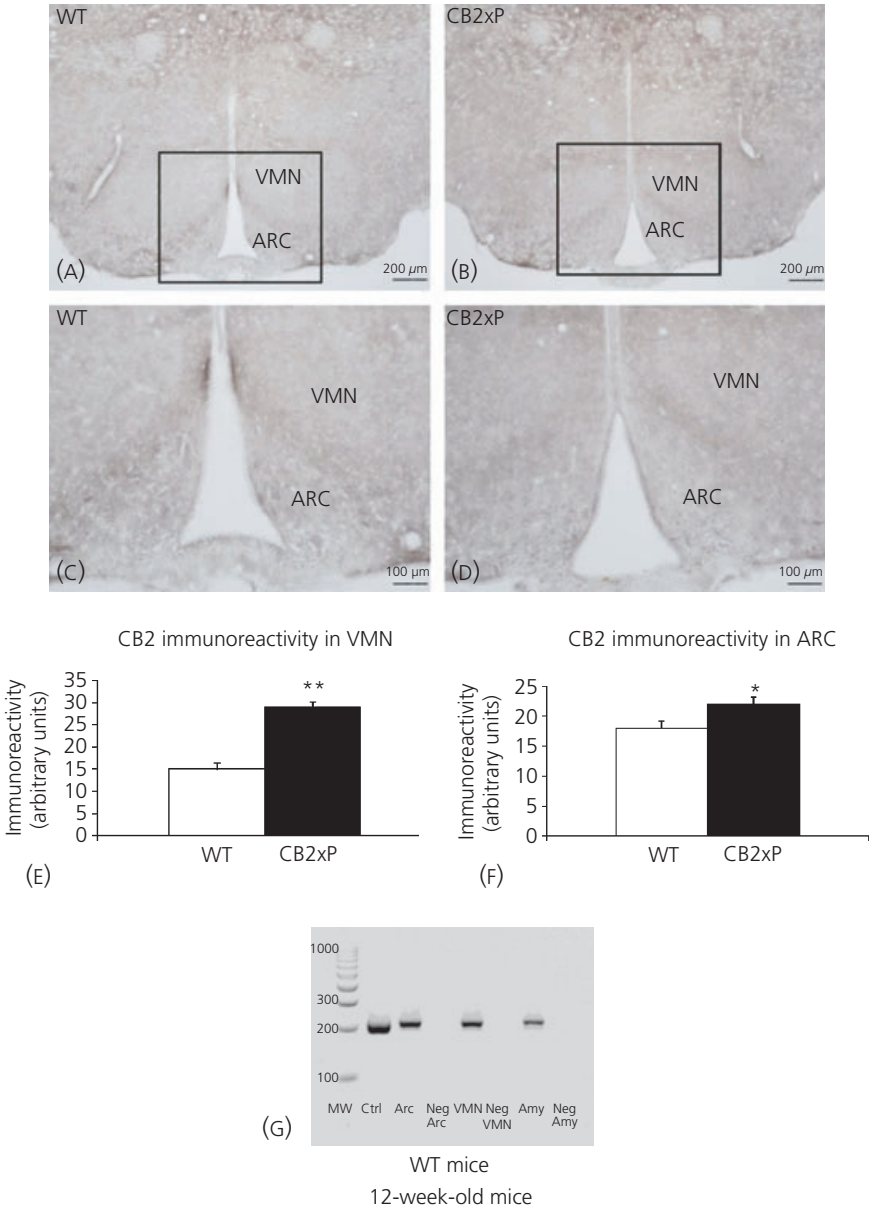

Fig. 1. CB2 receptor expression in the hypothalamus of wild-type (WT) and CB2xP mice. Immunohistochemistry of CB2 receptor in the hypothalamus from 12-week-old WT and CB2xP mice (A–D) and mRNA expression in the ventromedial nucleus of the hypothalamus (VMN) and arcuate nucleus (ARC) of WT mice (G). CB2 protein as well as CB2 mRNA are expressed in the hypothalamus of WT mice (A, C, G). Densitometric analysis of VMN and ARC immunoreactivity (E, F) showed increased CB2 protein expression in VMN and ARC from CB2xP versus WT mice. (C, D) Higher magnification views of the square areas in (A) and (B), respectively. Cnr2 gene expression was detected by RNA reverse-transcription (RT) to cDNA and subsequent polymerase amplification with CB2 specific primers. 'Ctrl' lane represents CB2 amplification from spleen, a tissue highly expressing CB2 receptor. 'Neg' lanes represent CB2 amplification from VMN, ARC and amygdala RT reactions in which the RT enzyme was omitted (n = 5 mice each genotype). The immunohistochemical images are representative of several slices. Scale bars are included in each image.

final extension step of 72 °C for 30 s in which fluorescence was acquired. Melting curves analyses and 1% agarose gel electrophoresis were performed to confirm that only a single product was amplified with the expected melting temperature molecular size. Gene expression was normalised to the expression of the reference gene histone cluster 1 (H2ab). The primers (Table 1) were designed based on NCBI database sequences of mouse reference mRNA and checked for specificity with BLAST software from NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers for cytosolic Phosphoenolpyruvate carboxykinase 1 (PEPCK) and glucose-6-phosphatase (G6Pase) were genome wide, bioinformatically validated primer sets commercially available from Qiagen (QuantiTect Primer Assay QT00114625 and QT00153013). CB2 receptor (Mm 00438286_m1) gene expression was measured using Taqman Gene Expression assay (Applied Biosystems, Madrid, Spain) as a double-stranded DNA-specific fluorescent dye and performed on the ABI PRISM 7700 Real Time Cycler (Applied Biosystems). The reference gene used was 18S rRNA, detected using Taqman ribosomal RNA control

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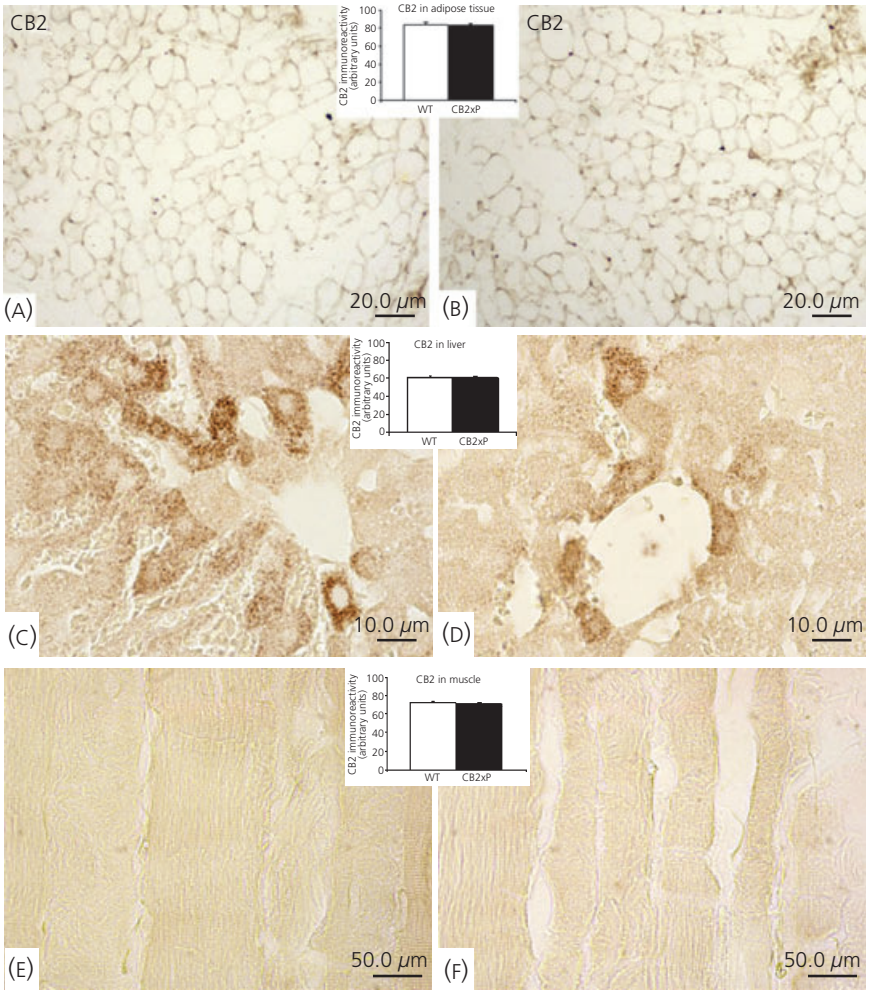


Fig. 2. Protein expression of cannabinoid CB2 receptor in metabolically-relevant tissues of wild-type and CB2xP mice. Immunohistochemistry and densitometric analysis of CB2 protein expression in 12-week-old mice showed no difference between the two genotypes in adipose tissue (A, B, inset), liver (C, D, inset) and muscle (E, F, inset) (n = 5 mice each genotype). Images are representative of several slices. Scale bars are included in each image.

reagents. Briefly, the data for each target gene were normalised to the endogenous reference gene, and the fold change in target gene abundance was determined using the $2^{-\Delta\Delta C_t}$ method (21). This quantitative method involves comparing the C_t values of the samples of interest with a control or calibrator, such as an untreated sample or RNA from normal tissue. The C_t values of both the calibrator and the samples of interest were normalised to an appropriate endogenous housekeeping gene. Gene quantification of hypothalamic neuropeptides and hepatic enzymes was carried out by using a standard curve run at the same time as the samples. The standards were a purified and quantified PCR product, serially diluted. Briefly, purified RNA from a tissue known to express the target gene was retro-transcribed to cDNA and PCR-amplified by using specific primers pair. The obtained PCR product was purified by using a high pure PCR product purification kit (Roche Diagnostic), quantified by 260 nm absorbance, and serially diluted to 10^{-6} pg/μl. An aliquot of the undiluted PCR product was subjected to a 1% agarose gel electrophoresis to check the fragment size and the absence of other contaminant fragments. Eight ten-fold dilutions (10^{1} to 10^{-6} pg/μl) were checked for optimal cycling on the iCycler system and five or six of them were selected for running the standard curve.

Statistical analysis

Data are expressed as the mean ± SEM. Comparisons were made using a two-tailed Student's t-test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GRAPHPAD PRISM software (GraphPad Software Inc., San Diego, CA, USA).

Results

Cnr2 mRNA overexpression level and behavioural characterisation in the CB2xP mice

Table 2 represents relative mRNA expression levels of the *Cnr2* gene in CB2xP mice compared to WT mice in discrete brain regions involved in different aspects of food intake, including areas involved in the hedonic properties of food. *Cnr2* gene is overexpressed in all these brain regions, with their increasing values ranging from 64% to 199% over

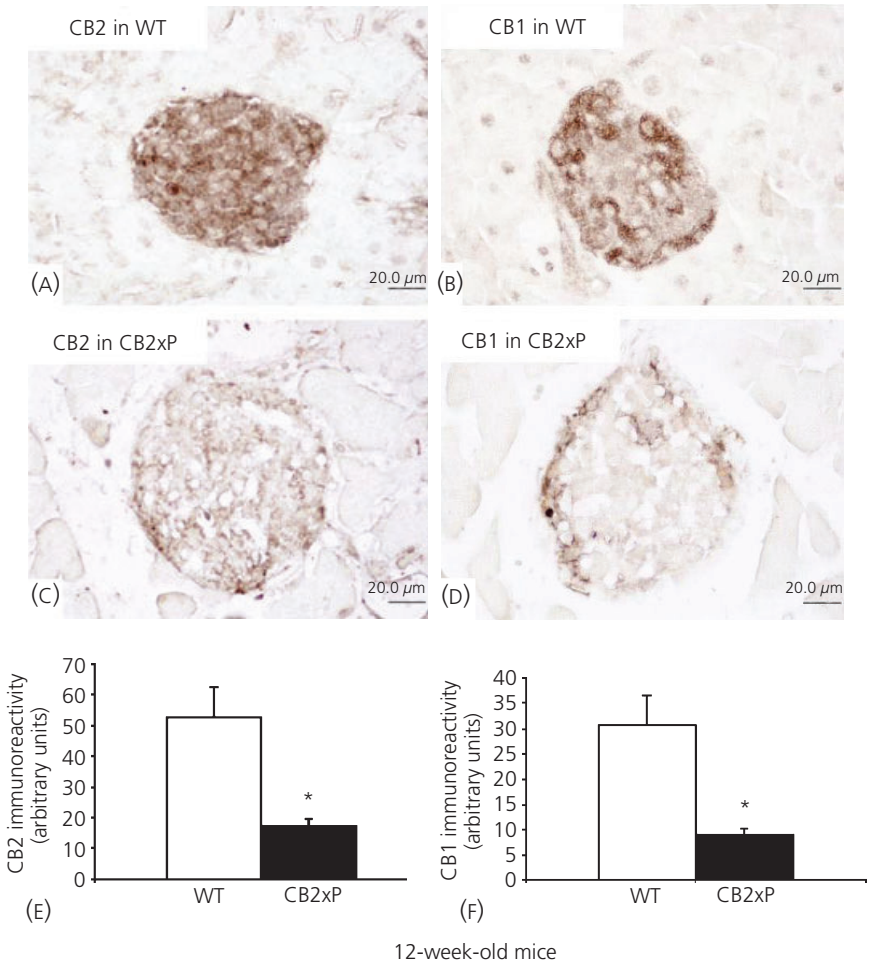


Fig. 3. Protein expression of cannabinoid CB1 and CB2 receptor in the endocrine pancreas. Immunohistochemistry and densitometric analysis of CB1 and CB2 protein expression in pancreatic sections from 12-week-old mice showed a decrease in both CB1 and CB2 receptors in the islets of Langerhans from CB2xP mice compared to wild-type mice (n = 5 mice each genotype). Student's t-test: *P < 0.05. Images are representative of several slices. Scale bars are included in each image.

those of WT mice. These mRNA changes are translated into increased expression of CB2 protein in the central nervous system (17). Table 3 summarises the main finding regarding behavioural characterisation of CB2xP in depressive and anxiogenic-like behaviours, conditions that potentially could influence food intake and glucose levels (17, 22). Taken together, the results presented in Table 3 show that CB2xP mice display no depressive symptoms or anxiogenic-like behaviours.

Cannabinoid CB1 and CB2 receptor expression in the hypothalamus and peripheral metabolically-relevant tissues

To assess putative changes in the expression of cannabinoid receptors in the hypothalamus and metabolically relevant tissues (i.e. adipose tissue, liver, muscle and endocrine pancreas), we performed immunohistochemistry and subsequent quantification by densitometry in sections from paraffin-embedded tissues of both WT and CB2xP mice. Figure 1 shows that CB2 receptors are expressed in the hypothalamus of WT mice at both the mRNA and protein level (Fig. 1A,C,G). Indeed, CB2 protein expression is increased in the hypothalamus of CB2xP versus WT mice. Immunoreactivity quantification of discrete hypothalamic areas reveals that CB2 protein is overexpressed in both the ventromedial nucleus of the hypothalamus (VMN) and the ARC. These data agree with those for mRNA levels (Table 2), suggesting that mRNA overexpression is translated into protein in the hypothalamus. Additionally, CB1 protein expression was unchanged in the hypothalamus (data not shown). Figure 2 shows no differences of CB2 protein expression in adipose tissue (Fig. 2A,B and inset), liver (Fig. 2C,D and inset) and muscle (Fig. 2E,F and inset) between WT and CB2xP mice. CB1 protein expression was also unchanged in these peripheral tissues (data not shown). By contrast, Fig. 3 shows a remarkable and statistically-significant decreased expression of both CB1 and CB2 receptors in the endocrine pancreas of CB2xP mice.

Effects of cannabinoid drugs on glucose tolerance in WT and CB2xP mice

To determine whether these changes in pancreatic CB1 and CB2 receptors have a functional impact on glucose homeostasis, the

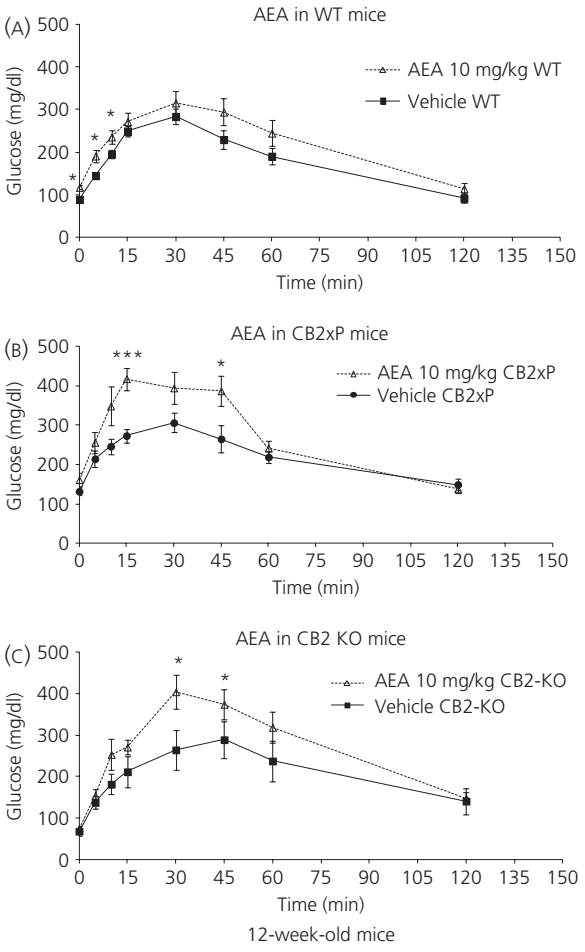


Fig. 4. Glucose tolerance test (GTT) in anandamide (AEA)-pre-treated wild-type (WT), CB2xP and CB2 knockout (KO) mice. GTT was performed in 12 h fasted WT, CB2xP and CB2 KO mice (12 weeks old) i.p. pre-treated with the endocannabinoid AEA, 10 mg/kg, 30 min before glucose overload (2 g/kg). AEA pre-treatment induced higher glucose intolerance in CB2xP (B) and CB2 KO mice (C) compared to WT (A) (n = 8–16 mice each genotype). Student's t-test: *P < 0.05, **P < 0.01.

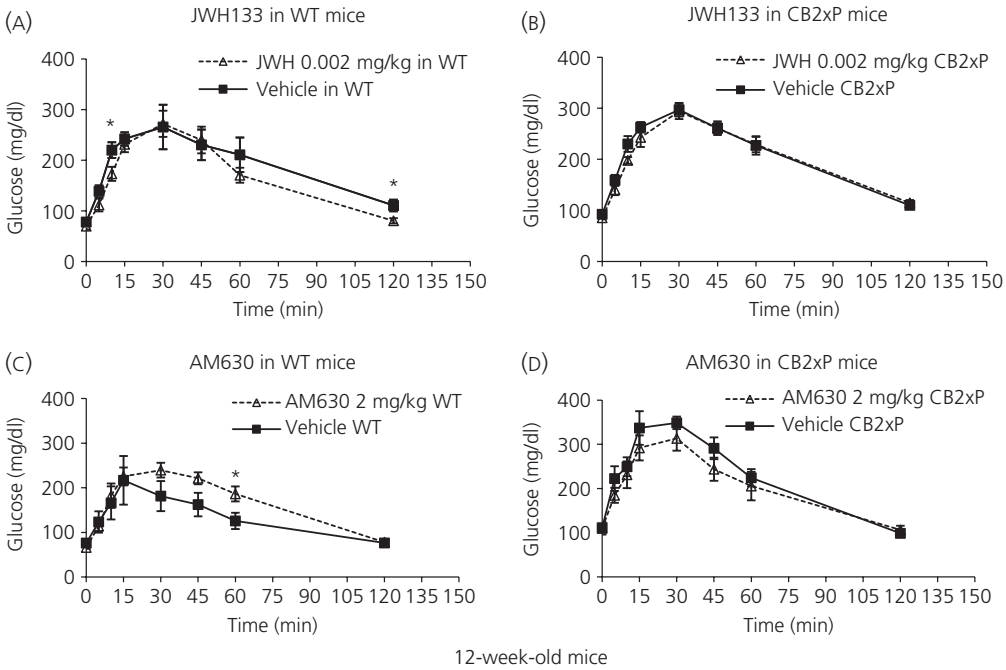
effects of AEA, an endocannabinoid, JWH133, a specific CB2 agonist and AM630, a specific CB2 antagonist, were evaluated on glucose tolerance after an acute glucose overload in both WT and CB2xP mice. Figure 4 shows that AEA induced glucose intolerance in CB2xP mice, with significant higher glucose levels than vehicle group at 15 and 45 min after glucose load (Fig. 4B), whereas there was a less pronounced effect on WT mice (Fig. 4A). Indeed, we also assessed the effects of AEA in mice lacking CB2 receptors. Figure 4(C) shows that AEA induced glucose intolerance in CB2-KO mice to a similar degree than in CB2xP mice. By contrast, JWH133 induced a moderate increase in glucose tolerance in WT mice, with significantly lower glucose levels than vehicle at 15 and 120 min after glucose load. This effect was blunted in CB2xP mice (Fig. 5A,B). Finally, the CB2 antagonist AM630 induced glucose intolerance in WT mice, with significantly higher glucose levels than vehicle at 60 min, an effect that was also blunted in CB2xP mice (Fig. 5C,D).

Fasting-induced feeding, weight gain and mRNA levels of ARC neuropeptides in WT and CB2xP mice

To assess putative changes of CB2xP mice in food intake and body weight, we measured cumulative food intake in fasted WT and CB2xP mice. Both groups had eaten the same 30 and 60 min after food presentation, although a decrease in food intake was evident at 120 min in CB2xP mice, as well as there being a strong tendency in the CB2xP mice to decrease food intake at 240 min (Fig. 6A). To evaluate the long-term effects of this increased sensitivity to satiety in CB2xP, we measured body weight at different ages. Figure 6(B) shows that, although body weight was similar in both genotypes at 12 weeks, 18- and 24-week-old CB2xP mice were leaner than WT mice. We also explored the mRNA levels of some neuropeptides involved in food intake control in the main hypothalamic area integrating hunger and satiety signals (i.e. the ARC). Thus, by quantitative RT-PCR, we measured POMC, neuropeptide Y and galanin. Figure 7 shows that mRNA expression of the anorexigenic factor POMC and the orexigenic factor galanin were decreased in CB2xP compared to WT mice, whereas neuropeptide (NPY) was unchanged between both genotypes, although showing a tendency to decrease in CB2xP mice.

Glucose homeostasis and related parameters

Basal glycaemia was measured in fasted WT and CB2xP mice at different ages. Basal glucose was higher in CB2xP than in WT mice at the three selected ages (Table 4). The glucose handling was investigated at these ages by performing GTT in animals from both genotypes. Accordingly, fasted WT and CB2xP mice were challenged with an i.p load of 2 g/kg glucose and blood glucose was monitored before and 5, 10, 15, 30, 45, 60 and 120 min after glucose injection. Figure 8(A) shows that there are no differences between genotypes in the GTT from 12-week-old mice but, by contrast, CB2xP mice aged 18 and 24 weeks displayed higher glucose values at almost all time points compared to WT mice of the same age (Fig. 8B,C). Quantification of AUC showed that 18- and 24-week-old CB2xP mice had a strong glucose intolerance (Fig. 8, inserts). We wanted to know whether this altered glucose tolerance involved changes in insulin secretion. For that purpose, we measured plasma insulin at different time points during the GTT in 15-week-old mice (also displaying glucose intolerance, data not shown). Figure 9(A) shows decreased insulin levels at 15, 30 and 45 min after glucose overload in CB2xP mice and, as a consequence, the insulin AUC was decreased (Fig. 9B). Given that glycaemia in the fasted state is highly regulated by hepatic glucose output, we also investigated the mRNA expression of cytosolic PEPCK, an enzyme involved in gluconeogenesis by converting oxaloacetate into phosphoenolpyruvate, and G6Pase, which catalyses the final step in gluconeogenesis, in the liver of 24-week-old WT and CB2xP mice. We could not detect differences in expression levels of any of these enzymes between genotypes (data not shown). Finally, to obtain a basic endocrine panel that could shed light on the metabolic phenotype displayed by CB2xP mice, we measured plasma levels of the key



10 **Fig. 5.** Glucose tolerance test (GTT) in JWH133- and AM630-pre-treated wild-type (WT) and CB2xP mice. GTT was performed in 12 h fasted WT and CB2xP mice (12 weeks old) i.p. pre-treated, 30 min before glucose overload (2 g/kg), with the CB2 agonist JWH133 (A, B) and the CB2 antagonist AM630 (C, D) at the indicated doses. JWH133 (0.002 mg/kg) and AM630 (2 mg/kg) increased and decreased, respectively, glucose tolerance in WT mice (A, C), with both effects being blunted in CB2xP mice (B, D) (n = 8–10 mice each genotype). Student's t-test: *P < 0.05, ~~**P < 0.01.~~

pancreatic hormones and the adipokines adiponectin and leptin (the former being an insulin-sensitising hormone and the latter being a satiety signal) in 24-week-old mice from both genotypes. Figure 10 shows that only glucagon was affected, with their levels being decreased in the CB2xP mice.

Discussion

It is well known that the endocannabinoid system, acting through cannabinoid CB1 receptors, exerts an important control on energy balance by both central and peripheral mechanisms (1, 23, 24). Pharmacological activation of cannabinoid CB1 receptors leads to weight gain by promoting food intake and adipogenesis, and by decreasing energy expenditure. Accordingly, CB1 blockade promotes weight loss and lipolysis and increases energy expenditure. The pharmacological effects of CB1 antagonists on obese mice have supported the development of a cannabinoid-based therapy for obesity and related diseases (25, 26). Indeed, rimonabant, a cannabinoid CB1 antagonist/inverse agonist, was marketed for the treatment of obese and overweight people with cardio-metabolic diseases. However, the reported side effects led to its removal from the market (27).

Endocannabinoid signalling is also channelled through cannabinoid CB2 receptors. These receptors were first described in the immune system and, indeed, they have been involved in obesity-associated inflammation (11). Currently, it is known that they are also expressed in other tissues, controlling diverse physiological processes. Among others, there is compelling evidence concerning the expression and function of cannabinoid CB2 receptors in the brain (13, 28–31). Together with a crucial role in modulating glial activation in response to nerve injury (15), central CB2 receptors appear to have an important role in response to stress, anxiety and depression (17, 22). Given the close relationship between drugs of abuse and depression, the role of CB2 receptors has also been explored in alcohol preference, as well as in opioids and cocaine consumption (32, 33). Furthermore, it has recently been demonstrated that brain CB2 receptors modulate the rewarding and locomotor-stimulating effects of cocaine, probably by engaging a dopamine-dependent mechanism (34). Interestingly, food and alcohol consumption has been investigated in parallel in a study analysing the behavioural effects of CB2 receptor activation. The specific antagonist AM630 inhibited food intake in C57Bl/6 mice, whereas its effect was opposite after 12 h of food deprivation (35). Similarly, an association between a *Cnr2* polymorphism and eating disorders has been described, thus suggesting a functional link between CB2 receptors and neural circuits controlling food intake and energy homeostasis (36). Taken together, these findings support a wider role of brain cannabinoid CB2 receptors than expected.

With these considerations in mind, we hypothesised that brain CB2 receptors may have a role in food intake and glucose metabolism. However, given the relatively low abundance of CB2 receptors in the brain that do not exclude the important role played by this receptor (31, 34), we decided to test our hypothesis in an animal model specifically overexpressing CB2 receptors at the central nervous system level. These transgenic mice were generated by using a murine PrP promoter that is specifically expressed in neurones and

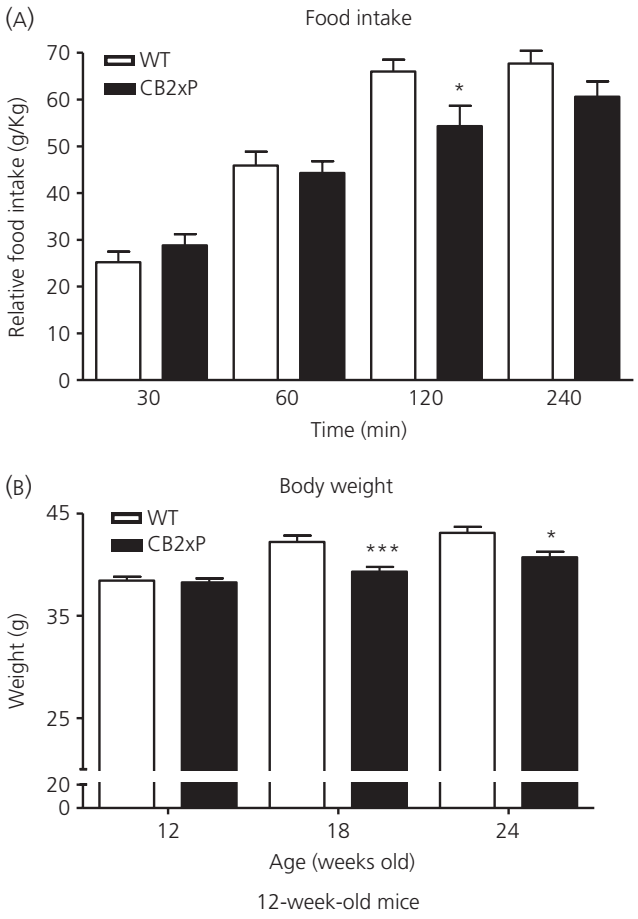


Fig. 6. Acute food intake and body weight follow-up in wild-type (WT) and CB2xP mice. Food intake was measured in 12 h fasted WT and CB2xP mice (12 weeks old) (A). The results are expressed as cumulative and relative to body weight food intake. One hundred and twenty minutes after food presentation, CB2xP mice had eaten less than the WT mice (n = 8 mice each genotype). Student's t-test: *P < 0.05. Body weight was measured in 12-, 18- and 24-week-old WT and CB2xP mice (B). Eighteen- and 24-week-old CB2xP mice weighed less than their age-matched WT mice, whereas there was no difference between genotypes at 12 weeks (n = 8–9 mice each genotype). Student's t-test: *P < 0.05, ***P < 0.001.

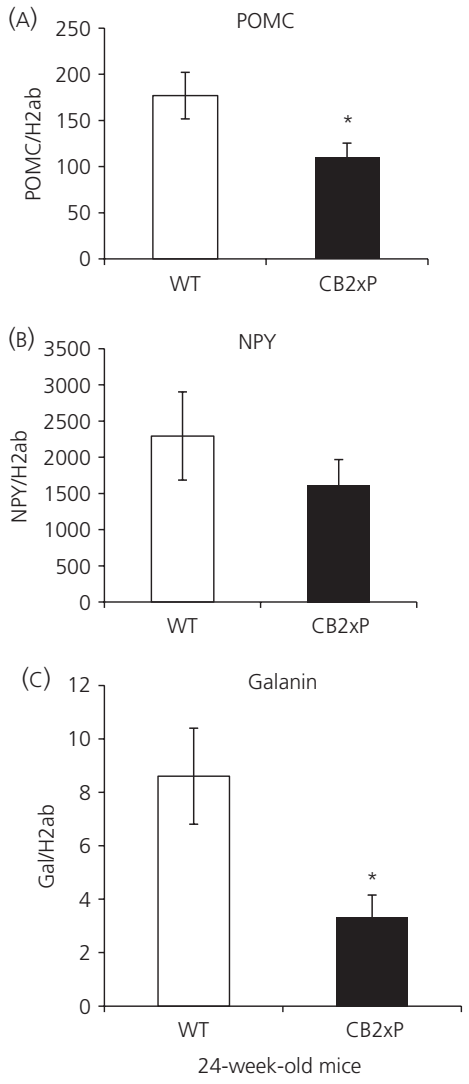


Fig. 7. mRNA expression of relevant neuropeptides in the hypothalamic arcuate nucleus in wild-type (WT) and CB2xP mice. Gene expression was measured in the arcuate nucleus of the hypothalamus in 24-week-old WT and CB2xP mice. Pro-opiomelanocortin (POMC) expression was decreased in CB2xP mice (A); neuropeptide Y (NPY) expression was unchanged, although showing a tendency to decrease (B); and galanin expression was decreased in CB2xP mice (C) (n = 5–7 mice each genotype). Student's t-test: *P < 0.05.

glial cells (16) and they have been validated and previously used to investigate the role of brain CB2 receptor in neuropathic pain (15), neuroprotection (18) and anxiety and depression (17, 37). These mice showed an increased CB2 mRNA expression of 157% and 126% in the ARC and VMN, respectively (two important hypothalamic regions controlling food intake), and a 100% increase in the ventral tegmental area, a brain structure related to the pleasant properties of food (Table 2). Moreover, mRNA analysis and immunohistochemistry showed that CB2 receptor is expressed in the hypothalamus of WT mice and, in CB2xP mice, the increased mRNA levels are translated into overexpressed CB2 protein in the hypothalamic VMN and ARC nucleus. These findings support the presence of CB2 receptors in the normal hypothalamus, and suggest that its overexpression could be a valuable tool for gaining new insights into its hypothalamic functions. However, the main limitation of this model should be mentioned. CB2xP mice overexpress CB2 receptor throughout the CNS. That means that, despite hypotha-

Table 4. Fasting Glucose Levels in CB2xP Mice in Adulthood.

	12 weeks	18 weeks	24 weeks
WT	77.88 ± 4.22	76.14 ± 4.76	66.44 ± 3.70
CB2xP	92.25 ± 4.91*	111.43 ± 4.18***	94.78 ± 4.38***

Twelve-hour fasted CB2xP mice of 12, 18 and 24 weeks exhibited higher plasma glucose levels than wild-type (WT) mice. Student's t-test: *P < 0.05, ***P < 0.001.

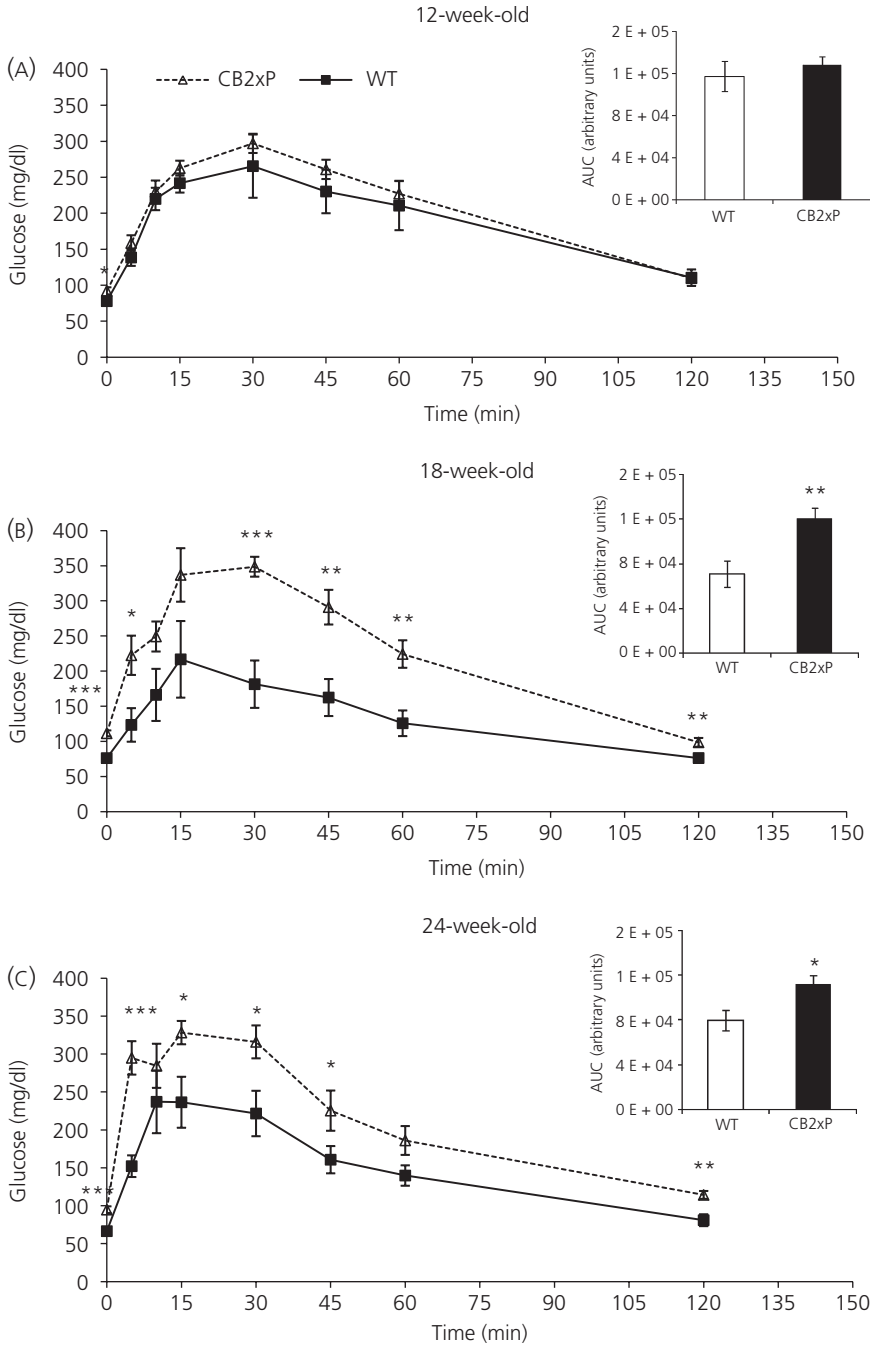


Fig. 8. Glucose tolerance test (GTT) in wild-type (WT) and CB2xP mice. GTT was performed in 12-, 18- and 24-week-old WT and CB2xP mice. Animals were food deprived for 12 h before the experimental procedure. Eighteen- and 24-week-old CB2xP mice showed glucose intolerance compared to their corresponding age-matched WT mice (B, C), whereas there was no difference at 12 weeks between both genotypes (A). The area under the curve was calculated for each age, displaying no significant changes between genotypes at 12 weeks (A, insert), and a statistically significant increase in CB2xP mice at 18 and 24 weeks (B, C, inserts) (n = 8 mice each genotype). Student's t-test: *P < 0.05, **P < 0.01 and ***P < 0.001.

lamic overexpression possibly unmasking its role in the normal hypothalamus, the overexpression in other brain areas could be a confounding factor. As such, the results reported in the present study represent the description of an endophenotype that can provide us with clues into the role of central CB2 receptors on energy homeostasis, although additional studies are needed to clearly establish the role of central CB2 receptors in energy balance.

The present findings show that overexpression of brain CB2 receptors: (i) decreases food intake, eventually leading to a lean phenotype and (ii) deregulates basal glucose levels, further altering

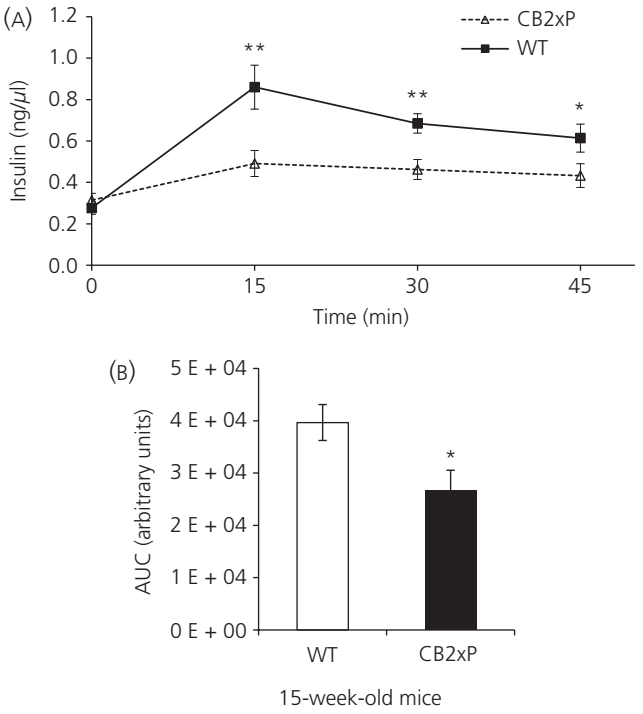


Fig. 9. Insulin plasma levels during the glucose tolerance test (GTT) in wild-type (WT) and CB2xP mice. GTT was performed in fasted 15-week-old WT and CB2xP mice. Tail blood samples were collected at the indicated time points and insulin plasma levels were measured by an enzyme-linked immunosorbent assay. Insulin plasma levels were lower in CB2xP than in WT mice at all time points checked after glucose overload (A). Insulin area under the curve (AUC) was accordingly lower in CB2xP versus WT mice (B) (n = 9–10 mice each genotype). Student's t-test: *P < 0.05 and **P < 0.01.

glucose homeostasis, with CB2xP mice showing chronic hyperglycaemia and glucose intolerance.

Regarding food intake, young food-deprived CB2xP animals (12 weeks old) ate less than their corresponding WT at 120 min after food presentation, suggesting that central CB2 overexpression changes sensitivity to hunger/satiety signals. This effect could not be the result of decreased locomotor activity, given that we could not find any difference in the total distance travelled during the open field test between both genotypes. Additionally, increased anxiety or depression leading to hypophagia in the CB2xP animals can also be discarded because CB2xP mice showed anxiolitic-like behaviours in a set of behavioural test such as the light dark box, elevated plus maze, tail suspension and novelty suppressed feeding, among others.

This acute finding in food intake was not correlated with body weight changes at this age but CB2xP mice eventually developed a lean phenotype, with their weights being statistically lower than those of WT mice at 18 and 24 weeks. Taken together, these findings suggest that the lean phenotype in CB2xP mice is a long-term consequence of the hypophagia shown at previous stages. These data fit well with a previous study showing that mice lacking CB2 receptors display increased body weight associated with increased food intake (12). Interestingly, these findings in $CB2^{-/-}$ mice were also age-dependent, with 8-week-old mice not displaying changes in body weight, whereas 6- and 12-month-old mice did. Accordingly, increased food intake was found that eventually led to an obese phenotype (12).

The decreased body weight in older CB2xP mice (18 and 24 weeks old) correlated with alterations in the mRNA levels of hypothalamic neuropeptides in 24-week-old CB2xP mice, with the anorexigenic peptide POMC and the orexigenic peptides galanin and NPY being decreased in the ARC. Thus, these genomic changes could potentially participate in the decreased food intake displayed by CB2xP mice, eventually impacting on body weight. However, in the present study, it is not possible to estimate the relative contribution of each of these genomic changes to the output hypothalamic signal in CB2xP mice. Although the decrease in POMC would contribute to increase food intake, the decrease in galanin and NPY would contribute to decrease it. Taken together, these results suggest that the central overexpression of CB2 receptors, including the hypothalamic overexpression described in the present study, alters the expression of hypothalamic neuropeptides involved in food intake, although the precise anatomical localisation of the CB2 receptors involved in this effect, the number of neuropeptides involved and the mechanisms underlying this interaction deserve more investigation. Thus, hypothalamic CB2 receptors could be involved in cannabinoid-mediated modulation of food intake. In the present study, we report that CB2 receptor is expressed in the VMN and ARC nucleus of the hypothalamus and it is known that cannabinoid CB1 receptors are expressed in GABAergic fibres entering the ARC, being capable of modulating GABA release onto POMC neurones (38). Similarly, pharmacological blockade of CB1 receptors by both rimonabant and AM251 has been reported to decrease NPY expression in the ARC (39, 40).

CB2xP mice displayed higher basal glucose levels than WT at all the ages tested, suggesting a role for central cannabinoid CB2 receptors in adjusting basal glucose concentrations. Intriguingly, the expression of CB1 and CB2 receptors in the endocrine pancreas, an anatomical position in which cannabinoid receptor has been suggested to play a role in glucose homeostasis (9, 20), was also altered in CB2xP mice, with their levels being decreased in mice centrally overexpressing this receptor. By contrast, there was no difference in the expression of CB1 and CB2 receptors in the liver, skeletal muscle and adipose tissue. This finding suggests an ability of glucose to modulate the expression of cannabinoid receptors in the endocrine pancreas, something that has been previously described *in vitro* for pancreatic CB1 receptors (41). Indeed, it strengthens the notion that cannabinoid receptors play an important role in islet physiology, as suggested previously (9). Previous studies have reported that CB1 agonism increases glucose intolerance, whereas CB1 antagonism decreases it (19). The effect of CB2 specific drugs was just the opposite, with CB2 agonism increasing glucose tolerance and CB2 antagonism decreasing it (20). Based on these findings, we attempted to pharmacologically dissect out the functional relevance of the cannabinoid receptor changes detected in CB2xP mice. For that purpose, we performed GTT on mice pretreated with cannabinoid drugs. AEA, which targets both CB1 and CB2 receptors, as well as vanilloid receptors, altered glucose homeostasis in CB2xP mice to a higher degree than in WT mice.

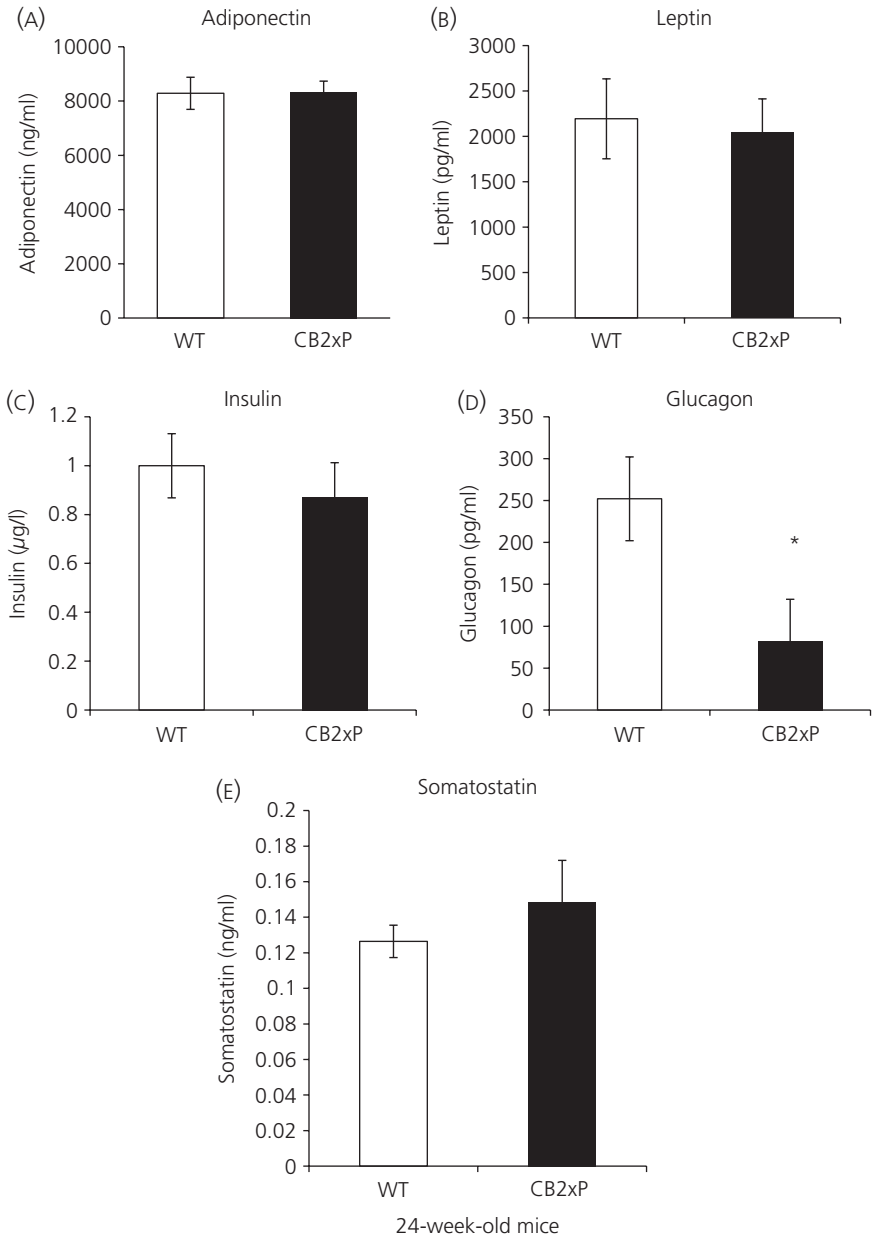


Fig. 10. Plasma levels of pancreatic hormones and adipokines in wild-type (WT) and CB2xP mice. Adiponectin, leptin, insulin, glucagon and somatostatin were measured in plasma samples from 12-h fasted WT and CB2xP mice (24 weeks). All these hormones were present at similar concentrations in both genotypes except glucagon, which showed decreased values in CB2xP mice (n = 8 mice each genotype). Student's t-test: *P < 0.05.

The CB2 down-regulation in islets of CB2xP mice could, at least partially, have a role in the AEA-induced glucose intolerance in CB2xP mice because CB2 KO mice also displayed a higher glucose intolerance than WT mice. Indeed, it is unlikely that pancreatic CB1 down-regulation is involved in this process because specific CB1 antagonism has been reported to increase glucose tolerance (19), and activation of vanilloid receptors has been reported to increase insulin secretion (42). Moreover, the CB2 agonist, JWH133, and the CB2 antagonist, AM630, decreased and increased, respectively, glucose values at certain time points in WT mice (20). Interestingly, these changes were missing in CB2xP mice, suggesting a functional role of CB2 receptors in these pharmacological effects. Taken together, these findings suggest that decreased pancreatic expression of cannabinoid receptors in CB2xP mice is functionally relevant.

Despite the higher basal glucose level displayed by 12-week-old CB2xP mice, they did not show the glucose intolerance that was eventually developed, even with the concomitant development of a lean phenotype with no changes in the plasma levels of important metabolic hormones such as adiponectin, leptin or insulin. The lack of hyperleptinaemia and hyperinsulinaemia in 24-week-old CB2xP mice suggests that glucose intolerance in CB2xP mice is not related to leptin and insulin resistance. However, the higher glucose levels

in the presence of the same levels of basal insulin suggest some kind of insulin insensitivity and/or a defect in a proper insulin secretion. Indeed, in the present study, we detected a defective insulin response in CB2xP mice after an acute glucose challenge, which suggests that alterations in glucose-induced pancreatic response are more related to the CB2xP glucose intolerance than decreased insulin sensitivity. However, a partial contribution of changes in insulin sensitivity cannot be ruled out. Interestingly, a previous study showed that CB2 receptor deficiency improves insulin sensitivity, with 12-month-old mice not developing insulin resistance and showing enhanced insulin-stimulated glucose uptake (12).

Regarding pancreatic hormones changes in CB2xP mice, we only detected a decrease in plasma glucagon levels compared to WT mice, whereas both insulin and somatostatin remained unchanged. Glucagon mobilises glucose from the liver and it has been reported to decrease food intake and increase energy expenditure (43). Thus, the decreased glucagon levels in the plasma of CB2xP mice cannot be the cause of the basal hyperglycaemia and/or the decreased food intake, although it may represent a compensatory mechanism aimed at partially counteracting the chronic increase of the basal glucose levels. Given that CB2 receptor activation has been reported to reduce the frequency of low-glucose-induced $[Ca^{2+}]_i$ oscillations in isolated islets of Langerhans from mice (44), it is feasible that there is a link between decreased glucagon levels and decreased CB2 expression in the endocrine pancreas. However, the lower glucagon levels are not likely to be a direct consequence of decreased CB2 expression in the endocrine pancreas, given that less signalling through CB2 receptors would result in increased glucagon secretion (44). By contrast, CB2 decreased expression could reflect a glucose-induced down-regulation of this receptor.

In conclusion, we have described the metabolic phenotype of mice centrally overexpressing CB2 receptors, with this overexpression leading to decreased food intake and body weight, increased basal glucose levels and, eventually, a lean phenotype with glucose intolerance. This paradoxical phenotype, which is strikingly similar to that of Asian populations (i.e. showing a lower body mass index and a higher incidence of type 2 diabetes), correlates with changes in the mRNA level of important hypothalamic neuropeptides, changes in the expression of cannabinoid receptors in the endocrine pancreas, altered sensitivity to cannabinoid-mediated modulation of glucose homeostasis and decreased plasma glucagon levels. However, there were no changes in basal plasma levels of important hormones such as adiponectin, leptin and insulin. Taken together, these results suggest a role for central cannabinoid CB2 receptors in modulating food intake, body weight and glucose homeostasis.

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References

1 Pagotto U, Marsicano G, Cota D, Lutz B, Pasquali R. The emerging role of the endocannabinoid system in endocrine regulation and energy balance. *Endocr Rev* 2006; **27**: 73–100.
2 Di Marzo V, Goparaju SK, Wang L, Liu J, Bátkai S, Járai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T, Kunos G. Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* 2001; **410**: 822–825.
3 Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Bátkai S, Harvey-White J, Mackie K, Offertáler L, Wang L, Kunos G. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest* 2005; **115**: 1298–1305.
4 Blüher M, Engeli S, Klöting N, Berndt J, Fasshauer M, Bátkai S, Pacher P, Schön MR, Jordan J, Stumvoll M. Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. *Diabetes* 2006; **55**: 3053–3060.
5 Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 2002; **54**: 161–202.
6 Mallat A, Lotersztajn S. Endocannabinoids as novel mediators of liver diseases. *J Endocrinol Invest* 2006; **29**: 58–65.
7 Starowicz KM, Cristino L, Matias I, Capasso R, Racioppi A, Izzo AA, Di Marzo V. Endocannabinoid dysregulation in the pancreas and adipose tissue of mice fed with a high-fat diet. *Obesity (Silver Spring)* 2008; **16**: 553–565.
8 Cavuoto P, McAinch AJ, Hatzinikolas G, Janovská A, Game P, Wittert GA. The expression of receptors for endocannabinoids in human and rodent skeletal muscle. *Biochem Biophys Res Commun* 2007; **364**: 105–110.
9 Juan-Picó P, Fuentes E, Bermúdez-Silva FJ, Javier Díaz-Molina F, Ripoll C, Rodríguez de Fonseca F, Nadal A. Cannabinoid receptors regulate Ca(2+) signals and insulin secretion in pancreatic beta-cell. *Cell Calcium* 2006; **39**: 155–162.
10 Bermúdez-Silva FJ, Suárez J, Baixeras E, Cobo N, Bautista D, Cuesta-Muñoz AL, Fuentes E, Juan-Pico P, Castro MJ, Milman G, Mechoulam R, Nadal A, Rodríguez de Fonseca F. Presence of functional cannabinoid receptors in human endocrine pancreas. *Diabetologia* 2008; **51**: 476–487.
11 Deveaux V, Cadoudal T, Ichigotani Y, Teixeira-Clerc F, Louvet A, Manin S, Nhieu JT, Belot MP, Zimmer A, Even P, Cani PD, Knauf C, Burcelin R, Bertola A, Le Marchand-Brustel Y, Gual P, Mallat A, Lotersztajn S. Cannabinoid CB2 receptor potentiates obesity-associated inflammation, insulin resistance and hepatic steatosis. *PLoS ONE* 2009; **4**: e5844.
12 Agudo J, Martin M, Roca C, Molas M, Bura AS, Zimmer A, Bosch F, Maldonado R. Deficiency of CB2 cannabinoid receptor in mice improves insulin sensitivity but increases food intake and obesity with age. *Diabetologia* 2010; **53**: 2629–2640.
13 Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD, Sharkey KA. Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* 2005; **310**: 329–332.
14 Brusco A, Tagliaferro P, Saez T, Onaivi ES. Postsynaptic localization of CB2 cannabinoid receptors in the rat hippocampus. *Synapse* 2008; **62**: 944–949.
15 Racz I, Nadal X, Alferink J, Baños JE, Rehnelt J, Martín M, Pintado B, Gutierrez-Adan A, Sanguino E, Manzanares J, Zimmer A, Maldonado R.

Crucial role of CB(2) cannabinoid receptor in the regulation of central immune responses during neuropathic pain. *J Neurosci* 2008; **28**: 12125–12135.
16 Castilla J, Gutiérrez-Adán A, Brun A, Pintado B, Salguero FJ, Parra B, Segundo FD, Ramírez MA, Rábano A, Cano MJ, Torres JM. Transgenic mice expressing bovine PrP with a four extra repeat octapeptide insert mutation show a spontaneous, non-transmissible, neurodegenerative disease and an expedited course of BSE infection. *FEBS Lett* 2005; **579**: 6237–6246.
17 García-Gutiérrez MS, Pérez-Ortiz JM, Gutiérrez-Adán A, Manzanares J. Depression-resistant endophenotype in mice overexpressing cannabinoid CB(2) receptors. *Br J Pharmacol* 2010; **160**: 1773–1784.
18 Ternianov A, Pérez-Ortiz JM, Solesio ME, García-Gutiérrez MS, Ortega-Álvaro A, Navarrete F, Leiva C, Galindo MF, Manzanares J. Overexpression of CB2 cannabinoid receptors results in neuroprotection against behavioral and neurochemical alterations induced by intracaudate administration of 6-hydroxydopamine. *Neurobiol Aging* 2012; **33**: 421.e1–16.
19 Bermúdez-Siva FJ, Serrano A, Diaz-Molina FJ, Sánchez Vera I, Juan-Pico P, Nadal A, Fuentes E, Rodríguez de Fonseca F. Activation of cannabinoid CB1 receptors induces glucose intolerance in rats. *Eur J Pharmacol* 2006; **531**: 282–284.
20 Bermudez-Silva FJ, Sanchez-Vera I, Suárez J, Serrano A, Fuentes E, Juan-Pico P, Nadal A, Rodríguez de Fonseca F. Role of cannabinoid CB2 receptors in glucose homeostasis in rats. *Eur J Pharmacol* 2007; **565**: 207–211.
21 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402–408.
22 García-Gutiérrez MS, García-Bueno B, Zoppi S, Leza JC, Manzanares J. Chronic blockade of cannabinoid CB(2) receptors induces anxiolytic-like actions associated to alterations in GABA(A) receptors. *Br J Pharmacol* 2012; **165**: 951–964.
23 Di Marzo V, Ligresti A, Cristino L. The endocannabinoid system as a link between homoeostatic and hedonic pathways involved in energy balance regulation. *Int J Obes (Lond)* 2009; **33**: S18–S24.
24 Bermudez-Silva FJ, Cardinal P, Cota D. The role of the endocannabinoid system in the neuroendocrine regulation of energy balance. *J Psychopharmacol* 2012; **26**: 114–124.
25 Cota D. CB1 receptors: emerging evidence for central and peripheral mechanisms that regulate energy balance, metabolism, and cardiovascular health. *Diabetes Metab Res Rev* 2007; **23**: 507–517.
26 Di Marzo V. Targeting the endocannabinoid system: to enhance or reduce? *Nat Rev Drug Discov* 2008; **7**: 438–455.
27 Bermudez-Silva FJ, Viveros MP, McPartland JM, Rodriguez de Fonseca F. The endocannabinoid system, eating behavior and energy homeostasis: the end or a new beginning? *Pharmacol Biochem Behav* 2010; **95**: 375–382.
28 Gong JP, Onaivi ES, Ishiguro H, Liu QR, Tagliaferro PA, Brusco A, Uhl GR. Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res* 2006; **1071**: 10–23.
29 Onaivi ES, Ishiguro H, Gong JP, Patel S, Perchuk A, Meozzi PA, Myers L, Mora Z, Tagliaferro P, Gardner E, Brusco A, Akinshola BE, Liu QR, Hope B, Iwasaki S, Arinami T, Teasenfitz L, Uhl GR. Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. *Ann NY Acad Sci* 2006; **1074**: 514–536.
30 Suárez J, Bermúdez-Silva FJ, Mackie K, Ledent C, Zimmer A, Cravatt BF, de Fonseca FR. Immunohistochemical description of the endogenous cannabinoid system in the rat cerebellum and functionally related nuclei. *J Comp Neurol* 2008; **509**: 400–421.
31 Onaivi ES, Ishiguro H, Gu S, Liu QR. CNS effects of CB2 cannabinoid receptors: beyond neuro-immuno-cannabinoid activity. *J Psychopharmacol* 2012; **26**: 92–103.
32 Ishiguro H, Iwasaki S, Teasenfitz L, Higuchi S, Horiuchi Y, Saito T, Arinami T, Onaivi ES. Involvement of cannabinoid CB2 receptor in alcohol preference in mice and alcoholism in humans. *Pharmacogenomics J* 2007; **7**: 380–385.
33 Onaivi ES, Ishiguro H, Gong JP, Patel S, Meozzi PA, Myers L, Perchuk A, Mora Z, Tagliaferro PA, Gardner E, Brusco A, Akinshola BE, Liu QR, Chirwa SS, Hope B, Lujilde J, Inada T, Iwasaki S, Macharia D, Teasenfitz L, Arinami T, Uhl GR. Functional expression of brain neuronal CB2 cannabinoid receptors are involved in the effects of drugs of abuse and in depression. *Ann NY Acad Sci* 2008; **1139**: 434–449.
34 Xi ZX, Peng XQ, Li X, Song R, Zhang HY, Liu QR, Yang HJ, Bi GH, Li J, Gardner EL. Brain cannabinoid CB(2) receptors modulate cocaine's actions in mice. *Nat Neurosci* 2011; **14**: 1160–1166.
35 Onaivi ES, Carpio O, Ishiguro H, Schanz N, Uhl GR, Benno R. Behavioral effects of CB2 cannabinoid receptor activation and its influence on food and alcohol consumption. *Ann NY Acad Sci* 2008; **1139**: 426–433.
36 Ishiguro H, Carpio O, Horiuchi Y, Shu A, Higuchi S, Schanz N, Benno R, Arinami T, Onaivi ES. A nonsynonymous polymorphism in cannabinoid CB2 receptor gene is associated with eating disorders in humans and food intake is modified in mice by its ligands. *Synapse* 2010; **64**: 92–96.
37 García-Gutiérrez MS, Manzanares J. Overexpression of CB2 cannabinoid receptors decreased vulnerability to anxiety and impaired anxiolytic action of alprazolam in mice. *J Psychopharmacol* 2011; **25**: 111–120.
38 Hentges ST, Low MJ, Williams JT. Differential regulation of synaptic inputs by constitutively released endocannabinoids and exogenous cannabinoids. *J Neurosci* 2005; **25**: 9746–9751.
39 Verty AN, Boon WM, Mallet PE, McGregor IS, Oldfield BJ. Involvement of hypothalamic peptides in the anorectic action of the CB receptor antagonist rimonabant (SR 141716). *Eur J Neurosci* 2009; **29**: 2207–2216.
40 Bakkali-Kassemi L, El Ouezzani S, Magoul R, Merroun I, Lopez-Jurado M, Errami M. Effects of cannabinoids on neuropeptide Y and β-endorphin expression in the rat hypothalamic arcuate nucleus. *Br J Nutr* 2011; **105**: 654–660.
41 Vilches-Flores A, Delgado-Buenrostro NL, Navarrete-Vázquez G, Villalobos-Molina R. CB1 cannabinoid receptor expression is regulated by glucose and feeding in rat pancreatic islets. *Regul Pept* 2010; **163**: 81–87.
42 Akiba Y, Kato S, Katsube K, Nakamura M, Takeuchi K, Ishii H, Hibi T. Transient receptor potential vanilloid subfamily 1 expressed in pancreatic islet beta cells modulates insulin secretion in rats. *Biochem Biophys Res Commun* 2004; **321**: 219–225.
43 Heppner KM, Habegger KM, Day J, Pfluger PT, Perez-Tilve D, Ward B, Gelfanov V, Woods SC, DiMarchi R, Tschöp M. Glucagon regulation of energy metabolism. *Physiol Behav* 2010; **100**: 545–548.
44 Juan-Pico P, Ropero A, Tuduri E, Quesada I, Fuentes E, Bermudez-Silva FJ, Rodriguez de Fonseca F, Nadal A. Regulation of glucose-induced $[Ca^{2+}]_i$ signals by cannabinoid CB1 and CB2 receptors in pancreatic α- and δ-cells within intact islets of Langerhans. *Obesity Metab* 2009; **5**: 20–28.

Author Query Form

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Dear Author,
During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.

Many thanks for your assistance.

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Q1	**AUTHOR: Please check author names and their affiliations.**	
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Q4	**AUTHOR: Please make reference in the legend to figures parts (A) to (F) appearing in the artwork to Figure 3..**	
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Q6	**AUTHOR: Supplied Tables (1 to 4) are in picture format. Therefore, we re-keyed tables from 1 to 4. Please check tables carefully.**	
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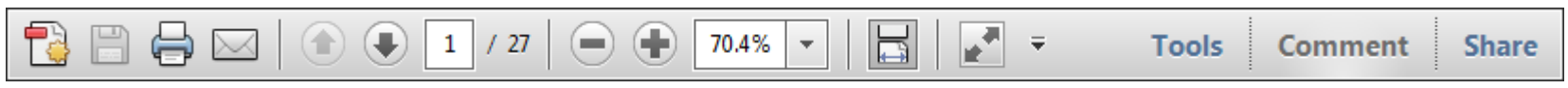
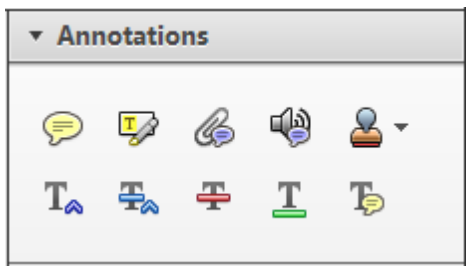
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

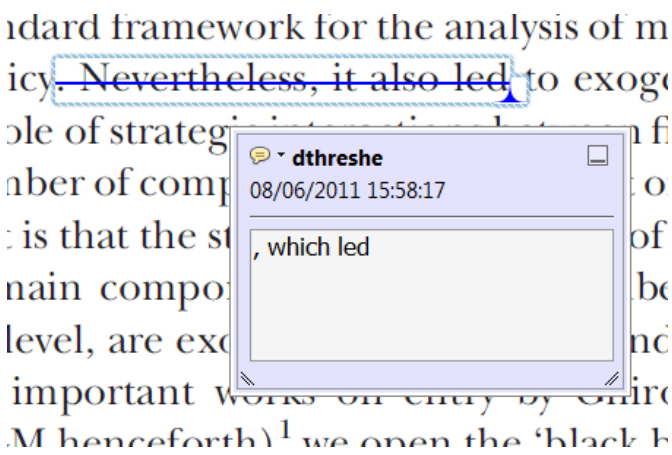


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

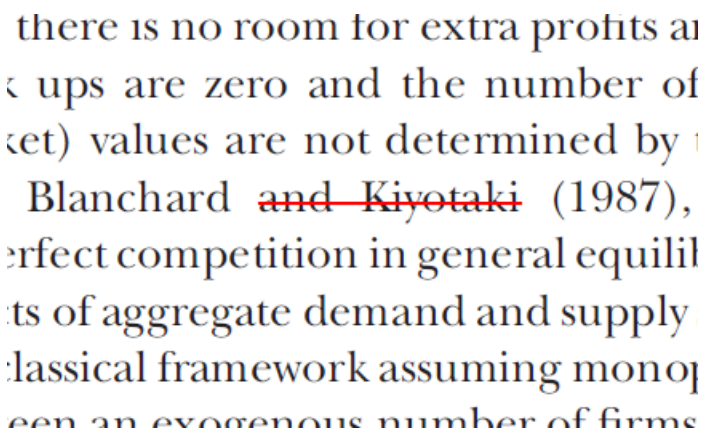


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

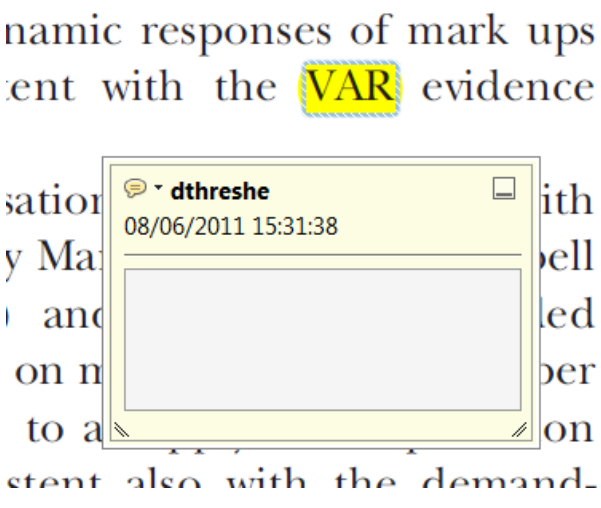


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

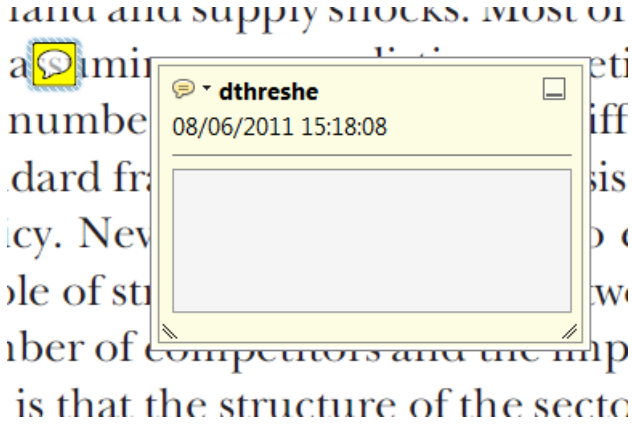


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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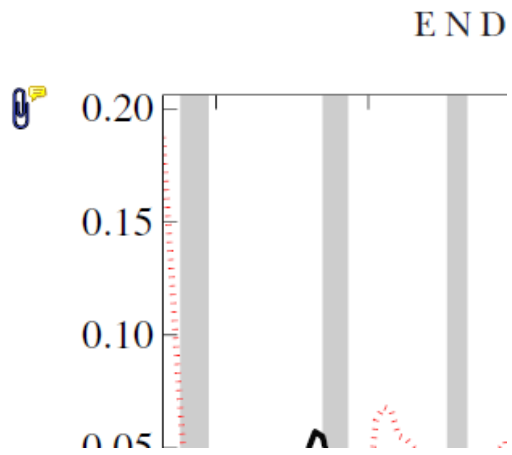
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

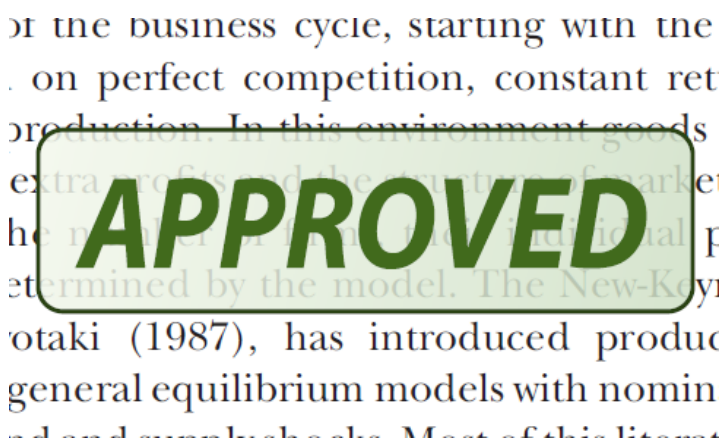


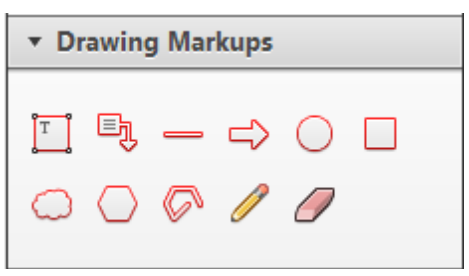
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

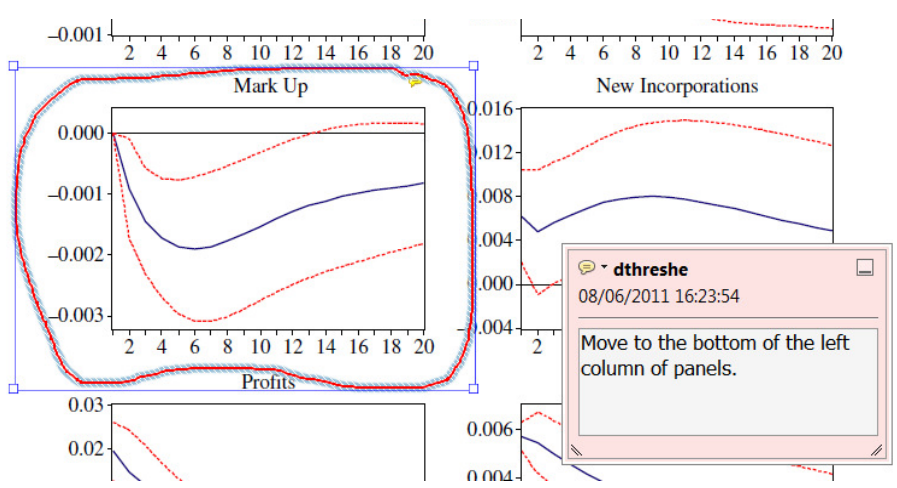
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).





7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: